

拟南芥 *Rboh* 基因家族成员的分子和功能学比较*孙旭东^{1,2**}, 胡向阳^{1,2}, 杨永平^{1,2}

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摘要: 活性氧 (reactive oxygen species, ROS) 在植物的信号转导中起着重要的作用。它们参与了植物的生长发育, 生物及非生物胁迫和细胞死亡等过程。最近的研究发现呼吸爆发氧化酶 (Respiratory burst oxidase homologues, Rboh) 是植物 ROS 的主要生产者。拟南芥 Rboh 基因家族由 10 个成员组成, 他们编码的蛋白包含 6 个跨膜结构域、以及 C 末端的 FAD 与 NADPH 亲水结构域和 N 末端的 2 个 Ca^{2+} 结合 EF 手性结构。本文通过聚类分析发现拟南芥 Rboh 基因家族成员的三个分枝具有高度的同源性, 这说明拟南芥 Rboh 家族成员间可能存在功能冗余。利用 RT-PCR 分析了各基因成员的时空表达特性, 并对整个家族成员的缺失突变体进行表型分析发现, 除了 *rbohC* 外, 都没有明显的表型变化, 这说明拟南芥 *RbohC* 可能在植物发育过程中具有特殊的功能。

关键词: NADPH 氧化酶; 呼吸爆发氧化酶基因; 聚类分析; RT-PCR; 功能冗余

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Molecular and Functional Comparisons of Reactive Burst Oxygen Species Gene Family in *Arabidopsis**SUN Xu-dong^{1,2**}, HU Xiang-yang^{1,2}, YANG Yong-ping^{1,2}

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Abstract: Reactive oxygen species (ROS) play a key role in cell signal transduction. They are involved in the regulation of growth, development, responses to abiotic or biotic stimuli and cell death. Recent studies identified respiratory burst oxidase homologues (RBOHs) as the key producers of ROS. The *Arabidopsis* genome contains 10 *Rboh* genes, that encode proteins with six transmembrane domains supporting two haem groups, FAD and NADPH hydrophilic domains in the C-terminal region and two calcium-binding domains (EF-hand) in the N-terminal region. In the present study, we investigated patterns of transcription systematically in *Arabidopsis* plants. Comparisons among amino acid sequences of ten *Rboh* genes revealed high degrees of identity among entire amino acid sequences of three groups, suggesting that some members of Rboh family might have redundant functions. With the except of *rbohC*, none of the loss-of-function mutants was found to display distinct phenotypes, suggesting that *RbohC* might have a specific function in plant development.

Key words: NADPH oxidase; Respiratory burst oxidase homologues (*Rboh*) genes; Phylogenetic analysis; RT-PCR; Redundant function

NADPH oxidases of NOX family exist in eukaryotes, and play essential roles in a variety of biological process,

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such as defense, signal transduction, and hormone synthesis. In plant, NADPH oxidases were designed as respiratory burst oxidase homologues (Rboh). Rbohs are plasma membrane proteins composed of six transmembrane domains supporting two haem groups, C-terminal FAD and NADPH hydrophilic domains and two N-terminal calcium-binding (EF-hand) domains. NADPH acts as a cytosolic electron donor to the extracellular O_2^- electron acceptor, which is reduced to O_2^- via FAD and two independent haems (Sagi and Fluhr, 2006). *Rbohs* genes belong to a small multigenic family, ten members in *Arabidopsis thaliana*, nine in rice and six in *Medicago truncatula*, composed of five groups of orthologous sequences (Marino *et al.*, 2011). Although several members of the *Rboh* gene family predicts a ubiquitous role (s) in proteins of the *Rboh* family, the molecular and cellular functions of all the members of this family are poorly understood.

Previous studies have been reported that some members of the *Rbohs* gene family, namely, *AtRbohD* and *AtRbohF* are shown to be involved in ROS production during pathogen infection (Torres *et al.*, 2002; Torres and Dangl, 2005; Chaouch *et al.*, 2012). It has already been reported that *AtRbohC/Root Hair Defectives (RHD2)* is functioned in root hair elongation (Foreman *et al.*, 2003). In tobacco, NtNOX has been shown to be functioned in normal growth of pollen tube (Potocky *et al.*, 2007). *Rboh*-dependent ROS production has also been linked to diffuse growth, such as the modulation of the cell wall loosening during seed germination, fruit ripening and root and hypocotyl elongation (Dunand *et al.*, 2007; Muller *et al.*, 2009).

In *rbohF* mutants, bacterial growth was increased, which was accompanied by delayed and radically decreased SA accumulation (Chaouch *et al.*, 2012). *rbohF* is essential for reprogramming plant metabolism and establishing an efficient resistance response during a susceptible interaction. Virus induced gene silencing of *NbRbohA* and *NbRbohB* in *Nicotiana benthamiana* compromised the resistance of plants a-

gainst the oomycete pathogen *Phytophthora infestans* and also reduced cell death response of the leaves (Yoshioka *et al.*, 2003). A potato (*Solanum tuberosum*) *StrbohA* is involved in the wound healing and resistance to natural microbial infections of potato tubers (Kumar *et al.*, 2007). Transient silencing of a barley (*Hordeum vulgare*) gene, *HvRbohA* decreased the penetration efficiency of the barley powdery mildew fungus, revealing that the *HvRbohA* protein facilitates fungal accessibility and hence promotes the development of powdery mildew (Trujillo *et al.*, 2006). During initiating trans-differentiation of epidermal transfer cells in *Vicia faba* cotyledons, two *Vicia faba* *Rboh* proteins, *VfRbohA* and *VfRbohC* generate a regulatory H_2O_2 signature, which directly polarized deposition of a uniform wall on which wall ingrowths form (Andriunas *et al.*, 2012).

To better understand the role of *Rboh* gene family in plant development, we conducted a comprehensive study of this family in *Arabidopsis*. Here, we report the results of the reverse transcription (RT)-PCR analyses, and the phenotypes of T-DNA insertion mutants of each *Rboh* members that has not been previously characterized. The possible roles of *Rboh* gene in plant growth and development might have redundant functions. With the except of *rbohC*, none of the loss-of-function mutants was found to display distinct phenotypes, suggesting that *RbohC* might have a specific function in plant development.

1 Materials and Methods

1.1 Plant materials and growth conditions

The wild type *Arabidopsis* (*Arabidopsis thaliana*) plants used in this study were the Columbia ecotype. T-DNA insertion alleles *rbohA* (CS862368), *rbohG* (CS833090) were obtained from the Syngenta T-DNA insertion collection (www.tmri.org). *rbohB* (dspm), *rbohC* (dspm), *rbohD* (dspm), *rbohE* (salk_150096), *rbohF* (salk_034674), *rbohH* (salk_136917), *rbohJ* (salk_050665) were obtained from the ABRC. *rbohI* (GK-H138) was obtained from GABI-KAT (www.gabi-kat.de).

Plants were grown on Murashige and Skoog (MS) agar medium (<http://www.phytotechlab.com>) or soil. After two days at 4 °C in darkness, plants were transferred to growth chamber at 200 $\mu\text{mol m}^{-2}\text{sec}^{-1}$ for 16-h light/8-h dark day/night cycle, at 20–22 °C constant temperature.

1.2 Genotyping

The genotype of each T-DNA insertion allele was determined by PCR of genomic DNA with using the three primers according to T-DNA Primer Design (<http://signal.salk.edu/tdnaprimers.2.html>). Wild type should get a product of about 1 000 bps, Homozygous lines will get a band of 400 bps, heterozygous lines will get both bands. The gene specific primers: *rbohA* (At5g07390): csLP GTGTTTGGAGGCACT-TGCTAC and csRP TTTAGCACTGAAACAGCCTCG; *rbohB* (At1g09090): 154Bdspm1 GAATAATGTA-ATTTAGTGAATGCCG and 74Bdspm11 ACAAATTC-GCTAGATTCAACCAT; *rbohC* (At5g51060): 62Cds-mpm1 TAGCTTCTCCATGTGACCGCG and 37Cds-mpm1 ATCTAAAGCTAGATGCCTTAGC; *rbohD* (At5g47910): 211Ddsmpm1: GTCGCCAAAGGAGGCGCCGA and 92 Ddsmpm1: GGATACTGATCATAGGCGTGGCTCCA; *rbohE* (At1g19230): salkLP GAGTTCGAGTTTCA-GTCACGC and salkRP ATGCAAGTTGATGAGAC-CTCG; *rbohF* (At1g64060): 171Fdspm1 CTTCG-ATATCCTTCAACCAACTC and 212Fdspm11 CGAA-GAAGATCTGGAGACGAGA; *rbohG* (At4g25090): csLP TTTTTGGCGTACCACTCAAAC and csRP CA-AAGAGTTTGGGTGATAGCG; *rbohH* (At5g60010): salkLP AGGTCTTGCAAAATGTGTTCCG and salkRP GCCGCTAAACCTAAACCAAAC; *rbohI* (At4g11230): gabiLP AATTGGGTAAACCATTGAAACC and gabiRP CGTGAGATCTTCTGGATCTGC; *rbohJ* (At3g45810): salkLP ACTTCAACATGCCAAAAATGG and salkRP TCAAAACAATTTCTATTTTGACGG. LBb1.3 (ATT-TTGCCGATTTCCGGAAC) for Salk lines. LB3 (TAG-CATCTGAATTTTCATAACCAATCTCGATACAC) for CS lines. dSpm1 (CTTATTTTCAAGAGTGTGGG-GTTTTGG) for dspm lines. gabiLB: (ATAATAAC-GCTGCGGACATCTACATTTT) for GABI lines. The conditions for the PCR reactions were: 35 cycles of

30 s at 94 °C, 30 s at 53 °C, and 60 s at 72 °C.

1.3 Chromosomal locations and analysis of duplication of genes in *Rboh* family

The chromosomal location of each member of the *Rboh* family was determined with the Chromosome Map Tool at TAIR (<http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp>). The location of each gene in relation to major chromosomal duplication events in the Arabidopsis genome was determined with tools provided at <http://wolfe.gen.tcd.ie/athal/dup>.

1.4 RT-PCR

Total RNA was extracted from various tissues from wild-type plants using TRizol[®] reagent (Invitrogen). For RT-PCR analysis, cDNA was synthesized from 2 μg of total RNA with an oligodT primer and M-MLV transcriptase (Transgen, China). Transcripts were quantified by RT-PCR analyses using 1/40th of the resulting cDNA as template. The housekeeping *ACT2* gene was used as internal standard. Triplet replicates of independent plant samples were performed. The gene-specific primers used were listed below: *RbohA*: CTGGCAAAGCTGCGCCGTTTC and CCGCTTACGTT-TTCCCTCCTCGC; *RbohB*: CGTGCCCAAAGGCAG-TGCAG and TTGCATGCAGCAAACGCGGG; *RbohC*: AGGTCCGAGCATCAAGCGGCTG and ACTCCTGCC-GGTGGTGGCTT; *RbohD*: GCGGCCATCCACGCAC-TCAA and CCGGAGACGTTATTCCGGCG; *RbohE*: CGCGCTCGAGCTTGTGTTC and TCCCGGTGAT-TCCTTCTGCACCT; *RbohF*: TCACAGCCAAAGGA-GCAGCTGAA and ACGAGGTCCAAGTATGTTGGC-TGT; *RbohG*: CCGACGGTAACGCTGCCTGG and CGTCTTCCGTAAGTCTACCATCCGA; *RbohH*: TT-GAGTGCCTCGGCGAACCG and CGGCGTTCTGTA-CCGCTCTGG; *RbohI*: TGCAGACGAGTTGTTCCGAC-GC and TGCTCGTTCTCGCAACCTCG; *RbohJ*: TG-ACGGGAGGCTTCCCAAGGA and ACGAGCCTGT-TCGCGGATGC. Every pair of primers span the exon3/exon5 region. PCR products were examined by separating on a 1.5% agarose gel.

1.5 Scanning electron microscopy

Leaves of wild-type plants and *rbohC* plants

were fixed and dehydrated as described previously (Sun *et al.*, 2013). The samples were observed and photographed under a scanning electron microscope (SEM, JSM-6360LV SEM; JEOL Ltd., Japan) at an accelerating voltage of 10 kV.

1.6 GUS staining

GUS staining was performed as described previously (Malamy and Benfey, 1997). The samples were examined using a Leica microscope, and images were captured using a Leica LM1000 camera.

2 Result

2.1 *Arabidopsis* genome contains 10 Rboh genes

There are ten members of reactive burst oxygen species (Rboh) genes in *Arabidopsis* genome. Fig. 1A shows a phylogenetic relationship between all of *Arabidopsis* Rboh proteins, which was deduced with neighbor-joining method. The *Arabidopsis* genome contains many putative paralogs that likely arose via segmental chromosome duplication events (Blanc *et*

al., 2000; Vision *et al.*, 2000). We investigated a possible relationship between the genetic divergence of members of the Rboh family and duplication events in the *Arabidopsis* genome. We examined the chromosomal locations and the duplicated segments in which genes of the AtRboh family are found, using data from the *Arabidopsis* Genome Initiative (2000) and from the report by Blanc *et al.* (2003) (Fig. 1B).

The *AtRboh* genes are distributed in four chromosomes, except chromosome 2. The search for such paralogs within the Rboh family by the use of the Paralogs in *Arabidopsis* program (<http://wolfe.gen.tcd.i.e./athal/dup>) revealed that *AtRbohA-AtRbohB-AtRbohC*, *AtRbohF-I*, and *AtRbohH-J* were located in the duplicated segments (Blanc *et al.*, 2003) and form paralogous gene pairs, respectively. The phylogenetic tree shown in Fig. 1A supports this notion. The comparison of the predicted amino acid sequences of the *AtRboh* genes also supported this notion

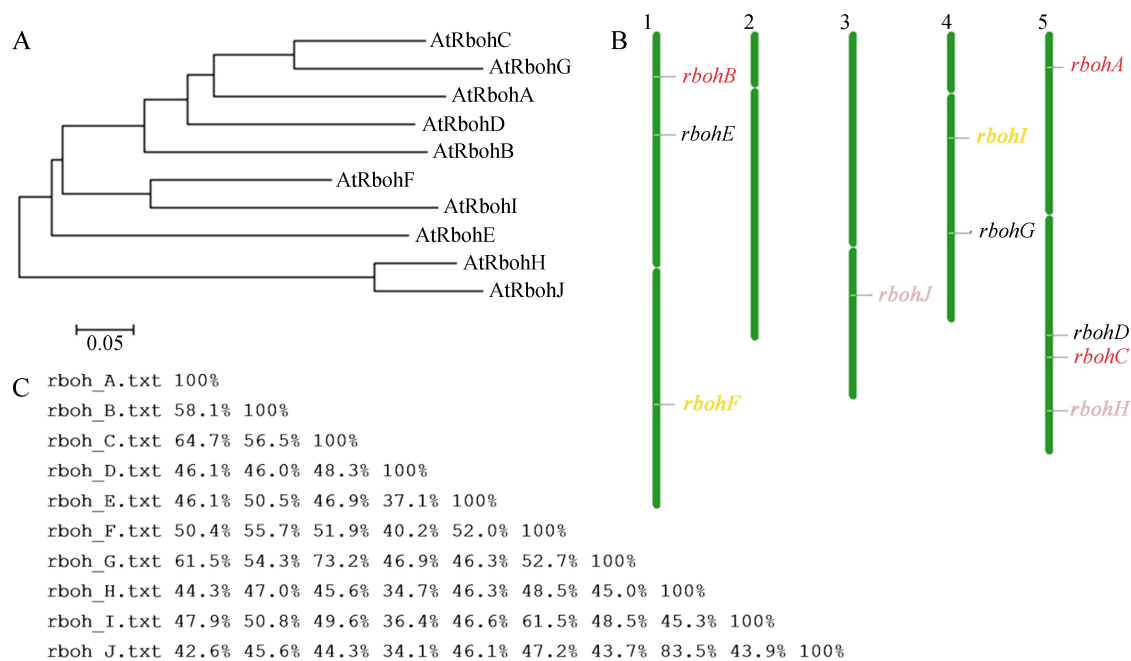


Fig. 1 A, The phylogenetic tree of *Arabidopsis* Rboh family proteins. An unrooted neighbor joining tree was generated using MEGA 6 neighbor joining software Bootstrap values (1,000 replicates) are shown. Bar (0.05) represents 5% amino acid substitutions per site per million years; B, Chromosomal positions and duplication of genes in the Rboh family in *Arabidopsis*. Green bars and white ellipses show the chromosomes and the positions of centromeres, respectively. The chromosome number is given at the top of each chromosome. Each set of putative duplicated genes is shown in a single color; C, Comparison of the predicted amino acid sequences of the *rboh* genes in *Arabidopsis*. Each number indicates the identity, as a percentage, between the predicted amino acid sequences of indicated proteins, as determined with the Maximum Matching program GENETYXMAC ver. 13

(Fig. 1C). The identity between *AtRbohA* and *AtRbohC* is 64.7%, *AtRbohF* and *AtRbohI* is 61.5%, *AtRbohH* and *AtRbohJ* is even up to 83.5%. These results suggested that members of *AtRboh* gene family might be duplicated in the distant past.

2.2 Expression of *AtRboh* genes

We performed RT-PCR to examine the patterns of expression of all *AtRboh* genes in a variety of *Arabidopsis* tissues. In all cases, primers spanning the predicted introns were used to distinguish between amplification of genome and amplification of cDNA. Expression were detected in 9 genes (Fig. 2).

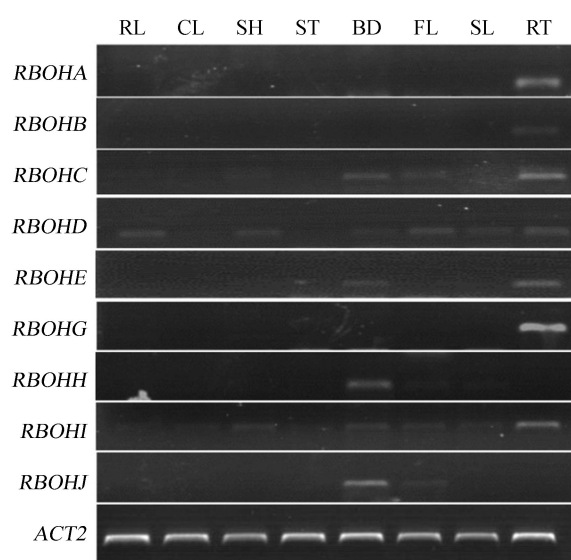


Fig. 2 RT-PCR analysis of the expression profiles of *Rboh* genes in wild type

RL, rosette leaves; CL, cauline leaves; SH, shoot tissue; ST, inflorescence stem; BD, floral buds; FL, open flowers; SL, siliques; RT, root. The former seven organ samples were prepared from 40-old-d flowering plants. The RT was 14-old-d seedlings grown on Murashige and Skoog agar medium

No expression was detected for *AtRbohF* in any of the tissues tested. This result indicated that the expression patterns of *AtRboh* genes were quite variable, with tissue or developmental stage-specific patterns (Fig. 2). Transcripts from *AtRbohA*, *AtRbohB* and *AtRbohG* were detected only in root. *AtRbohC* transcripts were detected in flower buds, open flowers and roots, low levels were also detected in shoot. *AtRbohD* transcripts were detected in all tissues ex-

cept cauline leaves and inflorescence stems. Whereas *AtRbohE* transcripts were expressed predominantly in roots, and also detected in flower buds, but not in open flower. Transcripts of *AtRbohI* were detected in all tissues examined, although at variable levels. Transcripts of both *AtRbohH* and *AtRbohJ* were primarily detected in flower buds, but faint bands were also amplified from open flowers.

2.3 Phenotypes of T-DNA insertion mutants of the *Arabidopsis Rboh* genes

For functional analysis of the *AtRboh* gene family, T-DNA insertion mutants of all *AtRboh* genes, were obtained from Syngenta, ABRC or GABI-KAT (Fig. 3).

Homozygous mutant plants were identified by PCR genotyping using gene-specific primers (Fig. 3). All of the single mutants except for *AtrbohC* showed no distinct phenotypes in terms of growth and development (Fig. 4).

However, *AtrbohC* showed to have lobed leaves (Fig. 5). Typically, the *Arabidopsis* leaf epidermis consists of interlocked jigsaw puzzle-shaped cells with protruding lobes and indentations (Xu *et al.*, 2003; Fu *et al.*, 2005).

Since loss-of-function mutant *AtrbohC* affected the leaf development, we suspected that the epidermis cell layer in *AtrbohC* mutants may display a phenotype. In order to assess the effect of *AtRbohC* on epidermal development, we analyzed cell shape in the epidermis of the leaf lamina of expanding rosette leaves from four-week-old *AtrbohC*. For consistency, a location midway up the length of the lamina and midway between the margin and the midvein of transgenic plants was chosen. The epidermis of WT laminae showed jigsaw puzzle-shaped cells (Fig. 6A). In contrast, the loss-of-function *AtrbohC* mutants displayed altered morphology of the leaf pavement cells. Cell shapes were often lacking lobes and necks (Fig. 6B, C, D). Transgenic plants carrying *ProRbohC* : *GUS* were prepared to localize *AtRbohC* expression more precisely. GUS staining was strong signal in the vascular tissues of the leaves, hypocotyl and roots in two-week-old transgenic plants (Fig. 7A, B).

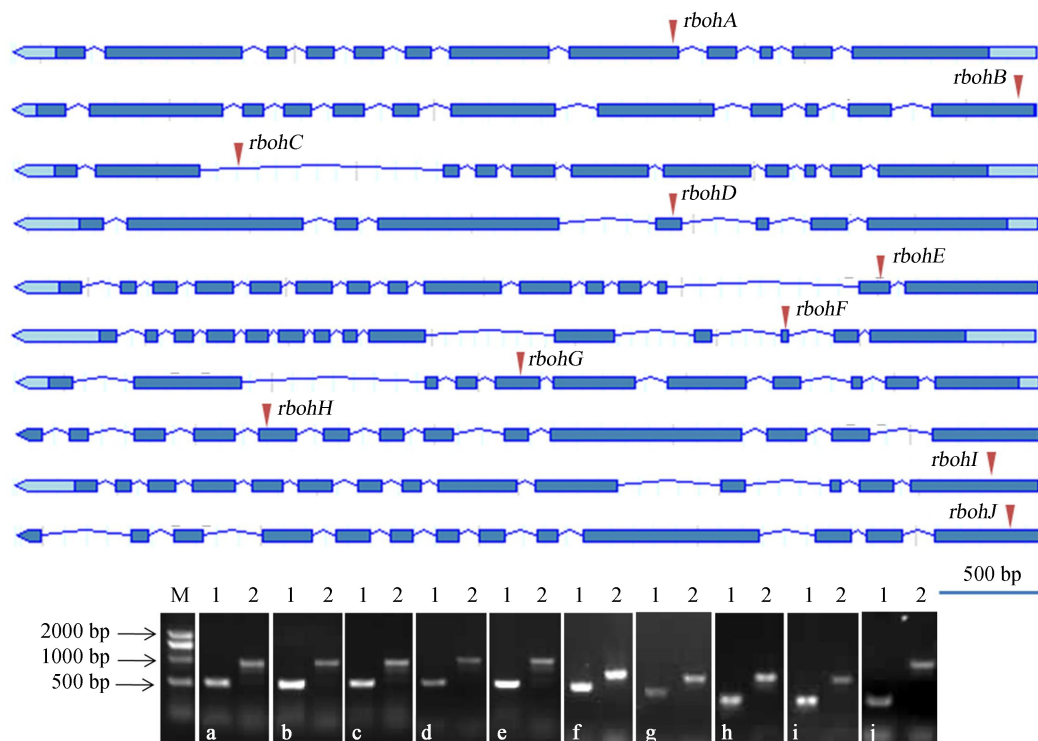


Fig. 3 Genomics structures of the *Arabidopsis* *Rboh* genes and locations of T-DNA insertions and Characterization of *rboh* mutants by PCR.

Exons are indicated by boxes, whereas introns are indicated by lines. Arrowheads indicate the T-DNA inserted localization

a, *rbohA*; b, *rbohB*; c, *rbohC*; d, *rbohD*; e, *rbohE*; f, *rbohF*; g, *rbohG*; h, *rbohH*; i, *rbohI*; j, *rbohJ*.

1, homozygote mutant; 2, wild type; M, DNA ladder marker

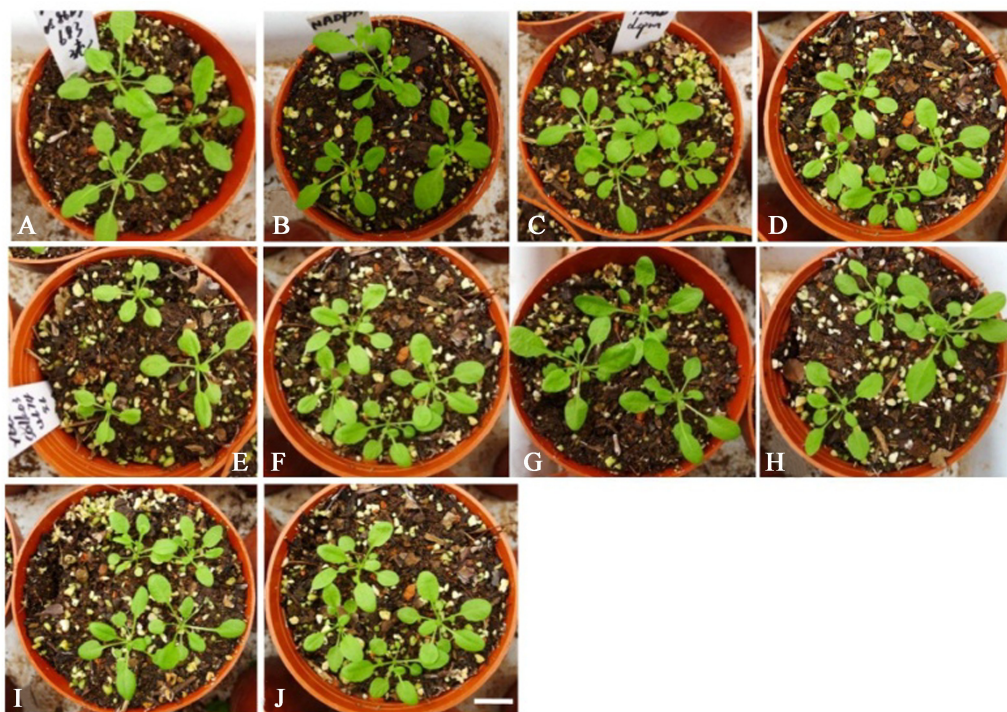


Fig. 4 Phenotypes of *rboh* mutants in *Arabidopsis*

A, *rbohA*; B, *rbohB*; C, *rbohD*; D, *rbohE*; E, *rbohF*; F, *rbohG*; G, *rbohH*; H, *rbohI*; I, *rbohJ*. J, wild type. bar = 1 cm

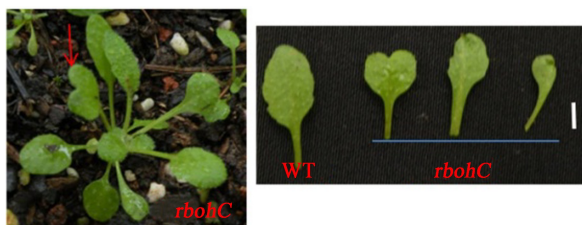


Fig. 5 Leaf phenotype of *rbohC* mutants. bar=0.2 cm

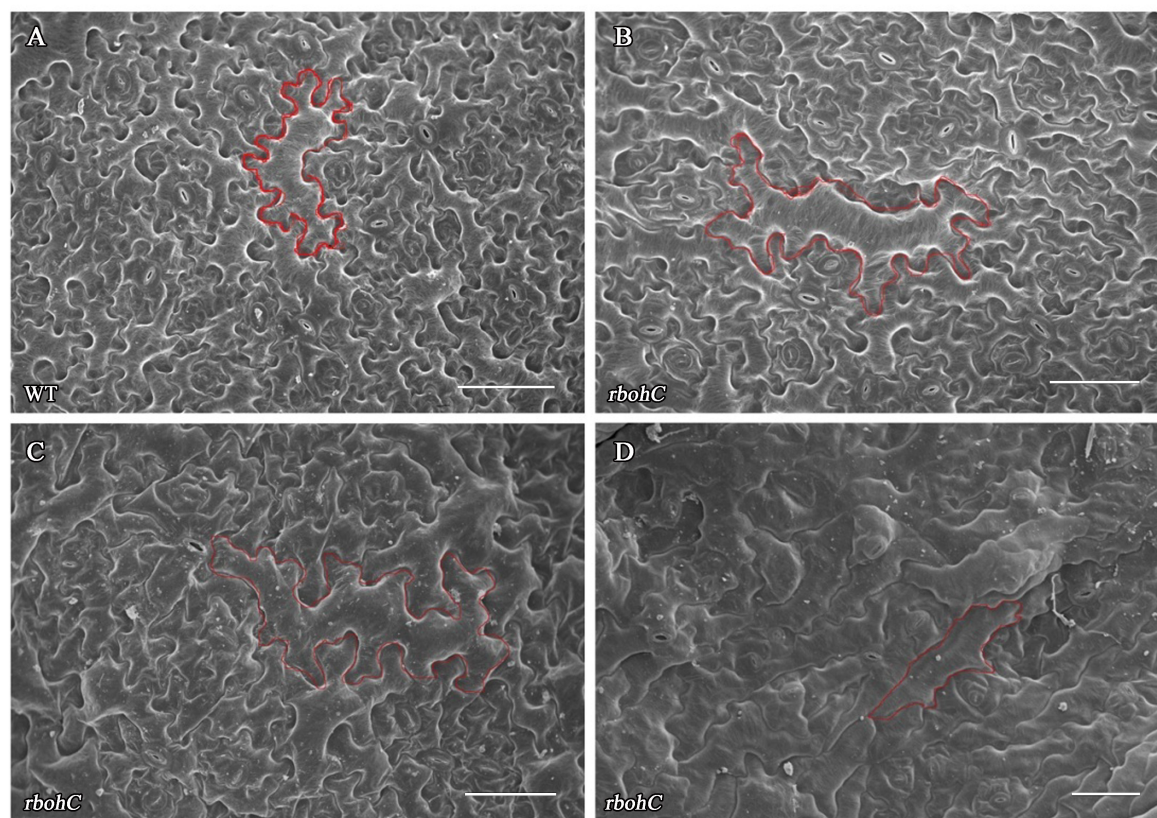


Fig. 6 Scanning electron microscope analysis of *rbohC* abaxial lamina epidermis. bar=50 μ m

A. jigsaw puzzle-shaped cells; B, C, D. cell shapes of lacking lobes and necks

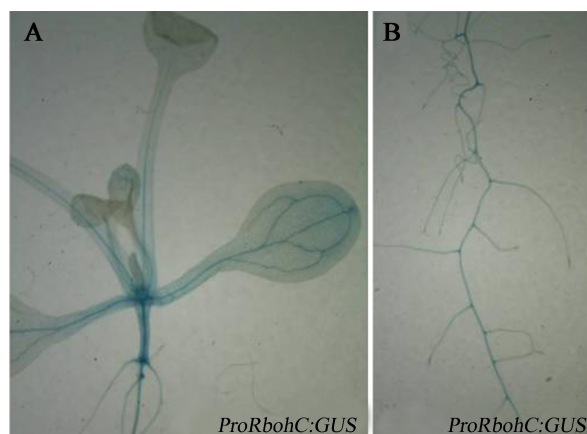


Fig. 7 GUS staining of a two-week-old seedling transformed with the *ProRbohC:GUS* construct

3 Discussion

In the present study, the transcripts of all members of the *AtRboh* gene family accumulate at different respective levels in *Arabidopsis* plants, and have a variety of transcriptional profiles in the whole plant. We also found three groups, with two or three members each, of *AtRboh* proteins with high degrees

of identity to one another in terms of their amino acid sequences. The members of the three groups were considered to be duplicated genes in each combination, suggesting evolutionary conservation.

In some groups, the members had similar transcriptional profiles (*AtRbohA*, *AtRbohB* and *AtRbohG*; *AtRbohH* and *AtRbohJ*). The members of each group might have overlapping functions in those organs in which they are expressed together. We obtained T-DNA insertion lines of all *Rboh* genes, and then we investigated the morphology of the aerial parts of these insertion lines. Except of *AtRbohC*,

there were no obviously differences between these insertion lines and wild-type plants. NtNOX, a tobacco NADPH oxidase, functioned in pollen tube growth (Potocky *et al.*, 2007). Transfection of tobacco pollen tube with NtNOX-specific antisense oligodeoxynucleotides resulted in decreased amount of NtNOX mRNA, lower NOX activity and pollen tube growth inhibition. The ROS scavengers and the NOX inhibitor diphenyleneiodonium chloride (DPI) also inhibited growth and ROS formation in tobacco pollen cultures. Exogenous hydrogen peroxide rescued the growth inhibition caused by NOX antisense oligodeoxynucleotides (Potocky *et al.*, 2007). AtRbohH and AtRbohJ, are NtNOX homologs, are specially expressed in floral buds. The *AtrbohH* and *AtrbohJ* single mutants, pollen tube tip growth was no obviously defect compared to the wild type; however, tip growth was severely impaired in the double mutant. In vivo imaging showed that ROS accumulation in the pollen tube was impaired in the double mutant. ROS production by AtRbohH and AtRbohJ is essential for proper pollen tube tip growth, and furthermore, that Ca^{2+} -induced ROS positive feedback regulation is conserved in the polarized cell growth to shape the long tubular cell (Kaya *et al.*, 2014). These results indicated that the members of *Rboh* gene family might have redundancy function in plant development.

RbohC/Root Hair Defective2 (RHD2) is one of the *Rboh* family members required for root elongation. *rhd2/rbohC* also displays impaired root hair elongation, due to defective ROS production and Ca^{2+} uptake, this result indicates that RHD2/AtRbohC is function in cell expansion of roots (Foreman *et al.*, 2003). Similar phenomena were observed in epidermis cell of rosette leaf in *rbohC* mutants. The animal NADPH oxidase gp91phox is regulated by Rac proteins, and similar proteins in plants called Rops (Rho-related GTPases from plants) have been shown to be involved in correct cellular expansion in the root elongation zone, the establishment of the tip-high Ca^{2+} gradient and root-hair growth (Molendijk

et al., 2001; Jones *et al.*, 2002; Wong *et al.*, 2007). Examination of GUS staining in leaves revealed that *RbohC* was expression in the developing leaves of two-week-old seedlings. These results suggested that *AtRbohC* maybe have similar function in the epidermal cell differentiation of developing leaves and root hair elongation through establishment of the Ca^{2+} gradient.

The *Arabidopsis Rboh* gene family has a diversified functions. *AtRbohH* and *AtRbohJ* display synergistic function during pollen tube development, whereas *AtRbohD* and *AtRbohF* are involved in ROS production in response to pathogens and ABA signaling. The accentuated phenotypes of the double mutants suggested that the different family members exist interaction in response to development and abiotic or biotic stress. Further identification of loss-of-function lines for these genes should define additional functions for plant development and response to stress. ROS generated by plant *Rboh* also functions in signaling pathway. Further characterization of the cross-talk between ROS signaling pathway and other signaling pathways may help to provide complex signaling networks in response to developmental cues or to environment.

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